REMARKS

This Amendment responds to the February 20, 2009 Office Action in which the Examiner rejected claims 1, 2, 5 and 10-13. Claim 14 was withdrawn pursuant to a restriction requirement. Reconsideration and reexamination are respectfully requested in view of the foregoing amendment and the following remarks.

The Examiner has rejected claims 1-2, 5, and 10-13 under 35 U.S.C. §102 (b), as anticipated by Cox. The Examiner asserts that Cox teaches saponin preparations from the bark of Quillaja saponaria Molina by weight of fraction A of Quil A and from 50-10% by weight of Fraction C of Quil A, where fractions Quil A human (QH) designated QH-A, QH-C ISCOMS are purified. The Examiner states that preparations of ISCOM matrix and protein-iscoms are made with QH703 or proteins, and the amounts of QH703 and Quil A confer different immunogenicity. The Examiner contends that at least two different saponin fractions of Quillaja saponaria Molina in separate iscom particles have immunomodulating activity, and therefore the Examiner concludes that the claimed invention is anticipated.

Applicant respectfully requests reconsideration, and maintain that the claimed invention is not anticipated by Cox. Cox discusses saponin preparations from the bark of Quillaja saponaria Molina (page 3, lines 24-26) comprising 50-90% by weight of Fraction A of Quil A and from 50-10% by weight of Fraction C

of Quil A (page 3, lines 23-25). Fraction A of the Quil A is designated QHA (page 7, lines 28-30) and Fraction C of Quil A is designated QHC (page 7, line 32- page 8, line 1-2). Cox further states that a particular combination of the Fractions A and C results in a saponin preparation with desirable properties, good ISCOM formation, and low hemolytic activity (page 8, lines 1-6, below Table 1). Thus, the individual saponin fractions QA and QHC are mixed to constitute QH703 (page 8, lines 6-9).

Cox also states that individual ISCOM matrix preparations can be made with pure QHA on one hand and pure QHC on the other hand. These two "pure" matrix formulations were compared with a matrix preparation made with the particularly preferred sapoinin preparation comprising 70% by weight of fraction A and about 30% by weight of fraction C (page 3, lines 27-30).

The overall conclusion from the data, as stated by the inventors, was that a mixture of A and C, roughly in the ratio of 7:3 (=7,0,3; or QH703) is an optimal ratio of purified saponins from which to form an ISCOM matrix or immunogenic ISCOMs. No formulation in Cox patent describes a formulation of QHA-matrix (or matrix-A) mixed with QHC-matrix (or matrix-C). Throughout Cox only single fraction matrices (e.g., QHA-matrix and QHC-matrix) made from a mixture of QHA and QHC saponins (QH703) are presented.

Cox simply does not render the claimed invention obvious, as the reference fails to teach how to construct a formulation having a mixture of preformed single fraction matrix particles as in the present invention. Neither can

it be gleaned from the Cox reference that such a modification would result in a significantly less reactogenic formulation with retained or even enhanced adjuvant properties.

The non-obviousness of this development can best be exemplified by the actions taken by CSL during recent years (Enclosure 1, reviewed in Drane et al., Expert Rev Vaccines 6 (5), 761-772). Drane states that the ISCOPREP 703, containing seven parts of fraction-A and three parts of fraction C has been abandoned, and that the new ISCOPREP used for matrix or ISCOMATRIX formulation no longer contains the fraction A. Thus, it contains fraction C saponins only.

The striking difference in reactogenicity of the 703 type of matrix particles made from mixtures of fraction A and fraction C saponins, that is, having matrix particles containing both saponin fractions compared with a mixture of pre-formed Matrix-A and Matrix-C particles is demonstrated in applicants' patent application in Example 4. Example 4 shows that the combined fraction A and C matrix seems more toxic than a matrix having only 10% fraction C, which is generally recognized as the more toxic saponin component (compared to fraction A). A mixture of one non-toxic component should, at least in theory, be less toxic than 100% with the toxic component. This finding is also corroborated in human clinical trials conducted by CSL (Drane et al., page 768, last six lines and page 769, three first lines), where it states the old ISCOMATRIX made by ISCOPREP 703 seem to be less well tolerated than the improved ISCOMATRIX consisting of

fraction C saponins only. The present invention is even less reactogenic than matrix particles produced from fraction C saponins alone, as demonstrated in Example 4. This is also evident from Enclosure 2 reactogenic tests.

In addition, the experiments presented in Enclosure 1 demonstrate that ISCOM formulations containing saponin fractions QH-A and QH-C formulated with the same particles exert toxicity in the same range as ISCOMs containing 100% of the toxic component QH-C alone. By contrast, the same amount of saponins mixed together but formulated in separate particles seems to be as free of side effects as a formulation containing 100% of the non-toxic component QHA, a totally unexpected and unpredictable result.

In addition, lethargy tests (presented earlier) show lethargy (described as side-effects and "not feeling well") and death tests performed with ISCOM particles comprising OVA antigen (egg albumin) and QHA in one ISCOM particle and OVA and QHC of Quil A in another particle (ISCOM-A+ISCOM-C 83:17) in Table 2 of Enclosure 1. This is compared with ISCOM -AC (83:17) with QHA and QHC in the same ISCOM particle thus corresponding roughly to the 703 mixture in the Cox patent. The lethargy score at 100 ng for ISCOM-C is 4, for ISCOM-AC (mixed as in the Cox patent) is 6 and according to the present invention ISCOM-A+ ISCOM-C is only 2.5. Enclosure 3, Fig. 1, shows that lethargy is lower for matrix mix according to the present invention (M-A(10) + M-A (1) and (2) respectively than for matrix containing QHC (Matrix C) only.

The cytotoxicity tests provide equally surprising results. While Matrix 703 (QHA and QHC in the same particle) has a LC_{50} value of around 18.7 in the cytotoxicity test (see Table 1 and Figure 2), Matrix MIX (QHA and QHC in different ISCOM matrix particles) has a LC_{50} value >240.

ISCOM formulations containing saponin fractions QHA and QHC formulated in the same particles display toxicity in the same range as ISCOMs containing 100% of the toxic component QHC alone. While saponins mixed together but formulated into separate particles do not display such side effects.

As mentioned above, CSL has discontinued its commercial product with fraction A and fraction C in the same ISCOM particle. Instead, they have reverted to the use of solely fraction C in ISCOM particles to avoid undesired side effects. The uncontroverted evidence shows, however, that when fraction A and fraction C are put into different ISCOM particles and thereafter mixed, they provide a better reactogenic effect, better lethargy test results, and less toxicity than even when fraction C is used alone. This unexpected result, applicants submit, undercuts the Examiner's assertion of obviousness.

Consequently, having fraction A and C in different ISCOM particles gives better results in several tests than when fraction C is used alone in ISCOMs, and also gives unexpectedly better results than when fractions A and C are put together in the same ISCOM particles, as was done in Cox. This unforeseen and unexpected result underscores the nonobviousness of the claimed invention.

Applicants respectfully request that the subject obviousness rejection be reconsidered and withdrawn.

If any fee is required in connection with the filing of the response, the Commissioner is authorized to charge the fee therefor to Deposit Account No. 03-3125. If a further extension of time is deemed required, applicants request such extension, and authorize the fee therefor to be charged to the foregoing deposit account.

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Respectfully submitted,

Robert D. Katz, Esq.
Registration No. 30,141
Cooper & Dunham LLP
Customer No. 23432
1185 Avenue of the Americas
New York, New York 10036
patentdocketing@cooperdunham.com
Tel. No. (212) 278-0400
Fax No. (212) 391-0525

Attorney for Applicants

Encl. 1

Review

ISCOMATRIX™ adjuvant for prophylactic and therapeutic vaccines

Debbie Drane†, Charmaine Gittleson, Jeff Boyle and Eugene Maraskovsky

The ISCOMATRIX™ adjuvant has antigen-delivery and -presentation properties, as well as immunomodulatory capabilities that combine to provide enhanced and accelerated immune responses. The responses are broad, including a range of subclasses of antibodies as well as both CD4⁺ and CD8⁺ T cells. A range of ISCOMATRIX vaccines (ISCOMATRIX adjuvant combined with antigen) have been evaluated in clinical trials. The results of these completed and ongoing studies indicate that the ISCOMATRIX adjuvant is safe and generally well tolerated and increases the vaccine immune responses.

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CONTENTS

†Author for correspondence
CSL Limited, 45 Poplar Road, Parkville, 3052 Victoria, Australia
Tel.: +61 3 9389 1902
Fax: +61 3 9389 2063
debbie.drane@csl.com.au

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The ISCOMATRIX™ adjuvant derives from the 'immunostimulating complex' or 'ISCOM', which was first described by Morein and colleagues in 1984 [1]. They subsequently showed that ISCOM-like structures could form in the absence of immunogen and called these structures ISCOM matrix [2]. The classical ISCOM™ vaccine required the incorporation of an amphipathic protein, typically a membrane protein, into a nascent structure that also required saponin, cholesterol and phospholipid for its formation. This not only restricted the types of proteins able to be formulated into a vaccine but also required a process that was complex and difficult to control. Our proprietary ISCOMATRIX adjuvant, which contains only a purified fraction of *Quillaia* saponin, cholesterol and phospholipid, can be made by a simple and robust manufacturing process. This can then be formulated with virtually any antigen to produce an ISCOMATRIX vaccine. A range of ISCOMATRIX vaccines have been tested in clinical trials and have been generally safe and well tolerated as well as immunogenic, generating both antibody and T-cell responses (for review see [3]). This makes the ISCOMATRIX adjuvant suitable for use in both prophylactic and therapeutic vaccines, which generally require antibody and cellular responses, respectively. In recent years, the focus has been on developing an improved ISCOMATRIX adjuvant to meet more easily the ever increasing regulatory standards for components of human vaccines, while maintaining the strong immune responses. The result is an optimized ISCOMATRIX adjuvant that is well defined, has minimal impurities and does not use any materials of animal origin. Additionally, improvements have been made to the methods of manufacture to ensure product can be manufactured reliably at any relevant scale. There has also been a much greater understanding of the mechanism of action of the ISCOMATRIX adjuvant.

Preparation & properties of ISCOMATRIX adjuvant

Composition

The ISCOMATRIX adjuvant contains saponin, cholesterol and phospholipid, typically in a phosphate-buffered saline (PBS) at pH 6.2. These components will be considered in turn.

Saponin, which comes from the bark of the *Quillaia saponaria* tree, is the potent immunomodulatory component. *Q. saponaria* saponins have been used for many years as adjuvants in animal vaccines, although these crude preparations are not suitable for human use owing to their toxicity and complexity [4]. More defined fractions of *Quillaia* saponin

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have been developed including QS21 [5], ISCOPREP™ 703 [6] and, more recently, ISCOPREP saponin. Apart from the obvious reduction in complexity, each of these fractions is selected to maximize adjuvant activity and minimize toxicity. CSL's prototype ISCOMATRIX adjuvant, from the mid 1990s, used ISCOPREP 703, which contained seven parts of fraction A and three parts of fraction C from *Quillaia* saponin. More recently, CSL has further developed a proprietary process for the fractionation of *Quillaia* saponin for use in its optimized ISCOMATRIX adjuvant. The resulting ISCOPREP saponin does not contain fraction A and thus avoids the complexity of ISCOPREP 703 and permits the characterization that is essential for materials to be used in human vaccines. The improved fractionation process also includes additional chromatography steps to eliminate impurities from the bark.

Cholesterol, which is synthesized from a plant precursor, interacts irreversibly with saponin. This interaction protects the saponin from hydrolysis and hence adds substantially to the stability of the ISCOMATRIX adjuvant [7]. Another critical feature of this interaction is that the ability of the saponin to interact with membranes is eliminated effectively. As a result, the hemolytic activity of saponin, which has been linked with the severe dose site reactions and other adverse events seen with QS21 [8], is essentially lost.

The phospholipid component contributes to the morphology and stability of the ISCOMATRIX adjuvant [9]. Phosphatidylcholine (PC) was traditionally used in ISCOM vaccines as it was identified as a component of the cell membrane required for consistent formation of ISCOM vaccines. However, commercially available PC is generally egg derived and is a complex mixture of molecules with different acyl chains. Dipalmitoyl-phosphatidylcholine was identified as the optimal phospholipid for the manufacture of ISCOMATRIX adjuvant for use in human vaccines as both acyl chains are the same and it is manufactured synthetically from plant-derived starting materials.

All of the components of the ISCOMATRIX adjuvant can be defined chemically and are either synthetic or derived from plant materials. In addition, there are no components or reagents used in the manufacturing process that are of animal origin. All raw materials are sourced from reputable suppliers and meet the stringent requirements for use in human vaccines.

Manufacturing processes

The manufacturing processes for both ISCOPREP saponin and ISCOMATRIX adjuvant are performed in accordance with good manufacturing practices in high-quality purpose-built facilities in the USA and Australia. The processes have been optimized extensively to ensure a consistent and effective product and extensive in-process and final testing is performed to guarantee compliance.

The ISCOMATRIX adjuvant manufacturing process is relatively simple as shown in FIGURE 1, and can be scaled easily to commercial production capacity as required. The cage-like

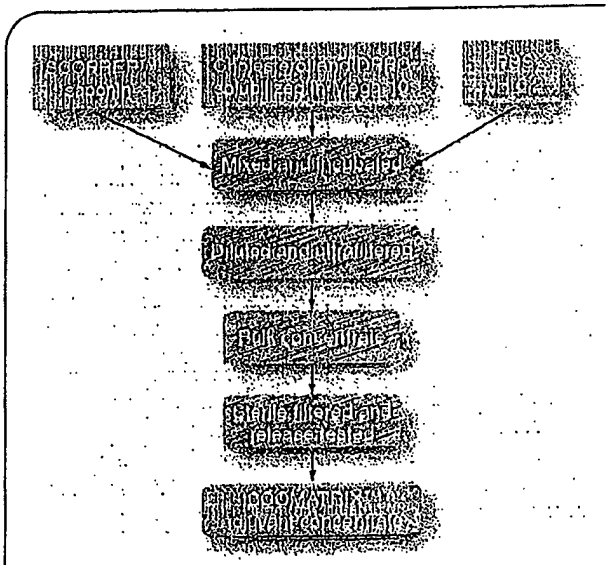

Figure 1. Flow chart of the ISCOMATRIX™ adjuvant manufacturing process.
Mega-10 (decanoyl-*N*-methylglucamide) is a nonionic detergent.
PBS: Phosphate-buffered saline.

structures of the ISCOMATRIX adjuvant form spontaneously when the components are mixed as described and exist in a low-energy state making them very stable. ISCOMATRIX adjuvant is provided as a sterile bulk concentrate at approximately 4 mg saponin/ml in PBS pH 6.2 and can be stored at 2–8°C where it is stable for at least 2 years. The release specification includes tests for identity, content, safety and biological activity but does not include a potency test as this would be performed on the vaccine formulation. Characterization, however, includes a range of immunological readouts in animal models, as well as further physicochemical analysis.

Physical properties

The particulate nature of the ISCOMATRIX adjuvant contributes to its antigen delivery capability. The cage-like structure and particle size, typically 40–50 nm in diameter, could be described as 'virus-like' enabling efficient phagocytosis by antigen-presenting cells (APCs). This feature is described in further detail later in this review under 'Mechanism of Action'.

The cage-like structure of the ISCOMATRIX adjuvant is best seen when examined by transmission electron microscopy (TEM). Techniques, such as cryoelectron microscopy and atomic force microscopy, have confirmed the spherical nature of the ISCOMATRIX adjuvant, as well as the particle size and ring-like subunit morphology as shown in FIGURE 2.

The molecular structure has not been fully elucidated, although the original model proposed by Kersten *et al.* in 1991 for the subunit structure has been generally supported by more recent studies using molecular dynamics (UNPUBLISHED DATA) [10]. It would appear that each of the ring subunits contains

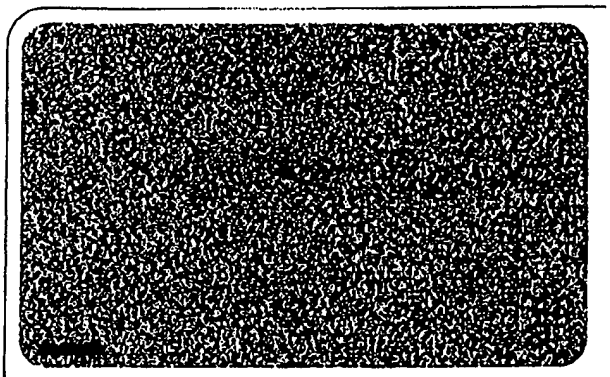

Figure 2. Thin-film cryoelectronmicrograph of ISCOMATRIX™ adjuvant. Bar = 100 nm. Image kindly provided by Ross Hamilton and Alex Hyatt.

saponin molecules inserted into a lipid bilayer in an extended conformation, such that both the triterpenoid core and acyl side chain interact with the bilayer in agreement with recent data using TEM by Myschik *et al.* [11].

The surface charge of the ISCOMATRIX adjuvant is approximately -20mV and is due to the glucuronic acid moiety in the saponin. This negative charge enables the ISCOMATRIX adjuvant to exist in solution as a stable colloid and can also contribute to formulation capabilities as described in the next section.

Both the particle size and high-performance liquid chromatography (HPLC) profile of its constituent saponin can be used to evaluate the stability of ISCOMATRIX adjuvant enabling a range of excipients and storage conditions including temperature to be easily evaluated. Studies show that ISCOMATRIX adjuvant remains stable in the presence of salts, sugars, denaturants, such as urea, and reducing agents (UNPUBLISHED DATA). Although stable in the presence of low concentrations of some detergents, ISCOMATRIX adjuvant can be broken down by higher concentrations of some detergents and studies would need to be performed for each detergent at the required concentration. A range of pHs have also been investigated and the ISCOMATRIX adjuvant is stable within the range pH 2–10. At low pH, it tends to aggregate, most probably due to a change in surface charge. At high pH, the ISCOPREP saponin undergoes alkaline hydrolysis that results in increased hemolytic activity and reduced immunological activity. This hydrolysis is enhanced at elevated temperatures, such as 50°C, although the ISCOMATRIX adjuvant has been shown to be stable at room temperature (25°C) for extended periods. Additionally, it can be stored frozen, repeatedly freeze–thawed, freeze dried and spray dried.

The physical properties of the ISCOMATRIX adjuvant, including its colloidal nature in solution and stability under a variety of conditions, permit formulation with a wide range of vaccine antigens. Generally, conditions can be found that meet the requirements of the antigen while maintaining the integrity of the ISCOMATRIX adjuvant structure.

Formulation methods

Formulation of ISCOMATRIX vaccines can be as simple as mixing the antigen and the ISCOMATRIX adjuvant and dispensing directly for use. In other circumstances, considerable effort may be required to optimize the conditions required to keep both the antigen and adjuvant in an optimally active form. The induction of antibody responses often requires the antigen to be maintained in a conformationally active form. Studies with ISCOMATRIX adjuvant have shown that simple mixing with antigens in a buffer compatible with the antigen results in an effective ISCOMATRIX vaccine. It should be noted, however, that the presence of detergents in the antigen buffer need to be evaluated because they may interfere with the integrity of the ISCOMATRIX adjuvant.

Induction of a cellular immune response appears to require the delivery of both the antigen and the ISCOMATRIX adjuvant to the same APC. To optimize this delivery, it is more efficient to have the antigen associated with the ISCOMATRIX adjuvant, which then requires, at least in animal models, less antigen to induce a strong cellular immune response. A number of methods have been developed to enable association of antigens with the ISCOMATRIX adjuvant. The simplest of these involves electrostatic binding of charged antigens [12]. The surface charge of standard ISCOMATRIX adjuvant is negative, which enables association with positively charged antigens. To accommodate negatively charged antigens, the surface charge of the ISCOMATRIX adjuvant can be altered by using different phospholipids or the charge of the antigen altered by adding positively charged sequences. The addition of charged sequences is particularly effective in peptide-based vaccines. Other novel methods that have been developed to enable association include the chelating ISCOMATRIX adjuvant, which contains a phospholipid with a chelating metal ion head group that can bind with a metal affinity tag, such as hexahistidine on an antigen [13].

Mechanism of action

ISCOMATRIX adjuvant possesses many integrated properties for the induction of immune responses. These include delivery and facilitation of antigen presentation, recruitment of immune cells to the draining lymph nodes via the induction of chemokines and cytokines and activation of the innate and adaptive immune systems (FIGURE 3). Understanding the relative importance of each of these features and how they integrate will be important for optimal use of the adjuvant in the clinic. Clearly, understanding the full details of this process at the molecular level is beyond the current state of scientific knowledge. However, as the range of immunological tools and reagents expands, so does the depth of our understanding of the immunological processes that underpin the potent properties of the ISCOMATRIX adjuvant. This is particularly relevant with regard to the capacity of the ISCOMATRIX adjuvant to induce $CD8^+$ T-cell responses. The adjuvant properties can be grouped into the broad categories of immunomodulation and antigen delivery [14] and are described in more detail later. Importantly, both the

immunomodulatory function, which can be attributed to the ISCOMATRIX adjuvant alone, and the delivery of antigen, which requires codelivery of the ISCOMATRIX adjuvant and antigen, are required for optimal CD8+ T-cell induction. This dual role and requirement is why ISCOMATRIX adjuvant is referred to as an integrated adjuvant. As shown in FIGURE 4, delay in delivery of the ISCOMATRIX adjuvant or antigen by 3 h dramatically reduces the immune response compared with coadministration as ISCOMATRIX vaccine.

Immunomodulation

The ISCOMATRIX adjuvant alone (i.e., in the absence of antigen), has been shown to have potent immunomodulatory effects at the level of the draining lymph node in sheep [15] and in mice (MANUSCRIPT IN PREPARATION). A characteristic of this priming event is that following subcutaneous administration of ISCOMATRIX adjuvant, there is an increased expression of proinflammatory cytokines, such as IL-6, IL-8 and IFN-γ. Concomitantly, the cellular output from the lymph node draining the injection site transiently (6–12 h) declines and then increases markedly above the resting levels 24–48 h later. Together, these observations demonstrate that ISCOMATRIX adjuvant is a potent immune modulator that both maximizes the number of low-frequency antigen specific cells entering the lymph node, thus increasing the potential for interaction with antigen or APCs, and orchestrates the local immune response via the induction of proinflammatory cytokines. Much of the early knowledge of the immunomodulatory capability of the ISCOMATRIX adjuvant has come from evaluations in animal models with ISCOM vaccines and, although this may be applicable, our more recent work has been focused strictly on the ISCOMATRIX adjuvant. For example, it was reported that cytotoxic T lymphocyte (CTL) responses were reduced dramatically in *IL-12*-knockout mice administered an ovalbumin (OVA) ISCOM formulation [16]. Although in these studies, lipopolysaccharide (LPS) contamination was very low (reported at 2 ng/dose), we have not been able to demonstrate a definitive role for IL-12 in CTL induction when these residual levels of LPS were removed (FIGURE 5A). The simplest explanation is that the CTL response in the earlier study combined the additive effect of IL-12-dependent LPS stimulation and IL-12-independent ISCOMATRIX adjuvant stimulation. Thus, it is critical that the analysis of which cytokines or chemokines are involved in the immune actions of ISCOMATRIX vaccines *in vivo* be conducted in the complete absence of LPS in order for clear interpretations to be made. Activation of the innate immune response in mice by ISCOMATRIX adjuvant does not appear to be mediated directly by Toll-like receptors (TLRs). We have shown that CTL responses are similar in wild-type and TLR4-deficient mice vaccinated with an ISCOMATRIX vaccine (FIGURE 5B). Interestingly though, there is significant overlap in the gene products upregulated by ISCOMATRIX vaccines and those of the TLR response genes. The detail of how the ISCOMATRIX adjuvant activates these TLR-independent innate immune responses remains to be elucidated and is the subject of ongoing experimentation but, as discussed later, may in part be due to the efficiency with which ISCOMATRIX vaccines are taken up and processed by APCs. *In vitro* exposure of human dendritic cells (DCs) to ISCOMATRIX adjuvant alone only weakly upregulated the expression on APCs of the major costimulatory molecule, CD86, or MHC II expression when compared with other agents, such as intact *Escherichia coli* or CD40 ligand [17]. However, these same maturation markers are dramatically upregulated on draining lymph node DCs (to levels similar to those seen with LPS) when ISCOMATRIX adjuvant is administered *in vivo* (FIGURE 6). This suggests that the types of cytokine cascades induced by ISCOMATRIX adjuvant *in vivo* cannot be reproduced *in vitro* and reveals a potentially important application for ISCOMATRIX adjuvant in the immunotherapy of chronic viral infections, such as herpes simplex virus, which downregulates the expression of MHC II.

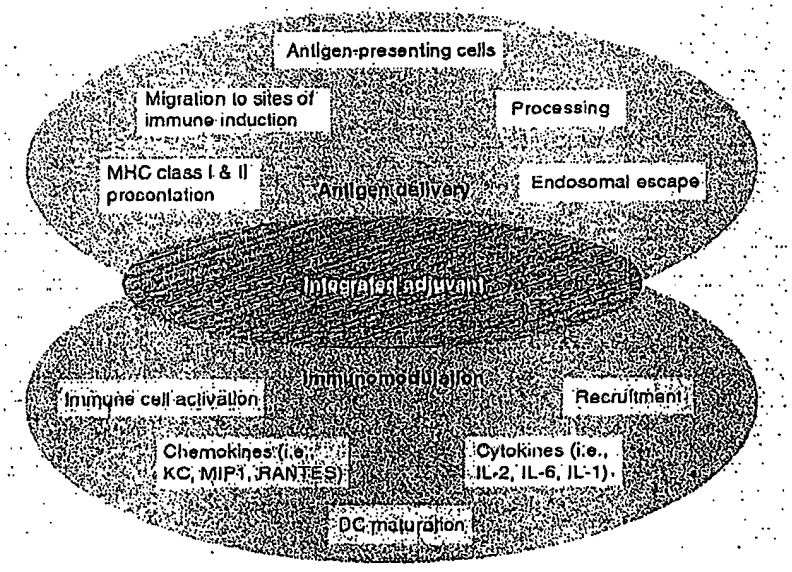

Figure 3. ISCOMATRIX™ adjuvant integrates both antigen delivery and immunomodulatory features.

Antigen delivery

To date, there is no evidence that cellular uptake of ISCOMATRIX adjuvant is mediated by specific membrane-bound receptors nor does it appear to bind to, and subsequently activate, APCs via interaction with TLRs (UNPUBLISHED OBSERVATION). However, it is important to keep in

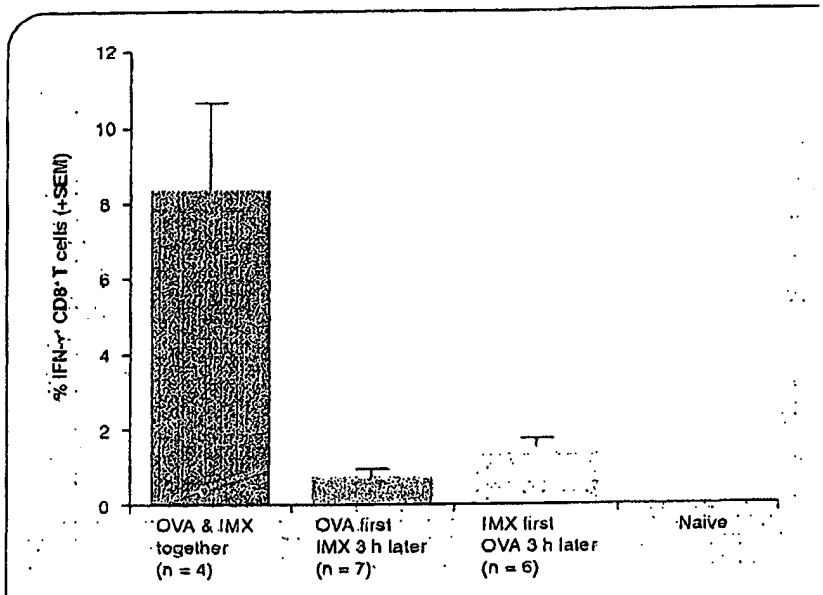

Figure 4. Both antigen delivery and immunomodulatory features of ISCOMATRIX™ adjuvant are required for optimal T cell immune induction. Groups of mice were immunized on day 0 and 7 with 5 µg of ISCOMATRIX adjuvant and 30 µg of OVA protein either together or sequentially in the same site 3 h apart as indicated. On day 14 the CD8+ T cell response to the OVA peptide SIINFEKL was assessed by intracellular cytokine staining.
IMX: ISCOMATRIX adjuvant; OVA: Ovalbumin.

mind that APC activation, although important, is clearly not the only adjuvant property required for the induction of cellular responses. This is particularly true for the induction of $CD8^+$ T cells, which also requires that antigen is delivered in such a way that it gains access to the MHC I antigen-processing pathway (FIGURE 3A). The possibility that the ISCOMATRIX adjuvant binds to an as yet unidentified receptor cannot be ruled out; however, it appears unlikely. Instead, it is possible that the hydrophobic nature of the ISCOMATRIX adjuvant facilitates its interaction with membranes at the cell surface and with subcellular organelles, such as endosomes, facilitating translocation of antigen into the cytosol. Furthermore, because of its particulate nature, the ISCOMATRIX adjuvant is effectively targeted to, and taken up by, APCs. Being typically 40–50 nm in diameter, ISCOMATRIX adjuvant cage-like structures are similar in size to the viral pathogens that the immune system has evolved to eliminate. Consistent with this, the optimal particle size for inducing CTL responses was in the 40–50 nm range [18]. Once bound to APCs, the antigens in ISCOMATRIX adjuvant are rapidly taken up and processed for both MHC I and MHC II presentation [19].

Antigen processing

One of the greatest challenges for the development of subunit vaccines that are capable of inducing $CD8^+$ CTL responses has been the requirement to deliver antigen to the cytoplasm so that it can gain access to the MHC I processing pathway. The ISCOMATRIX adjuvant achieves this goal and, at the same time, is able to access MHC II antigen processing. This latter is perhaps not all that surprising, given that this pathway is primarily fed by cellular uptake of exogenous antigens via phagocytosis. The explanation for why ISCOMATRIX adjuvant targets the MHC I pathway so effectively is not immediately obvious, although recent studies in this area have made considerable progress toward providing an answer to this question. Most MHC I-binding peptides are generated in the cytosol as side products of the degradation of misfolded proteins, a process that primarily occurs in the proteasome. A subset of the resulting peptides are translocated across the endoplasmic reticulum (ER) membrane by a dedicated peptide transporter, and then loaded onto peptide-receptive MHC I molecules in the ER and transported to the cell membrane. Robson *et al.* have shown *in vitro* using mouse cells and an OVA ISCOMATRIX vaccine that bone marrow-derived DCs, but not macrophages or naive B cells, prime antigen-specific $CD4^+$ and $CD8^+$ T cells [20,21].

Similarly, *in vitro* studies using human cells with an NY-ESO-1 ISCOMATRIX vaccine have shown that only CD1c blood DCs and monocyte-derived DCs are capable of presenting epitopes on both MHC I and MHC II, whereas plasmacytoid DCs are limited to MHC II presentation [19]. Detailed examination of antigen processing of NY-ESO-1 in these human monocyte-derived DCs has shown that for MHC I epitope generation, ISCOMATRIX adjuvant targeted NY-ESO-1 to a fast, proteasome-independent cross-presentation pathway, whereas soluble NY-ESO-1 protein or NY-ESO-1 immune complexes targeted a slow, proteasome-dependent pathway. Both NY-ESO-1 in the form of immune complexes, which are generally regarded as an efficient way to load DCs for MHC I processing [19], and NY-ESO-1 ISCOMATRIX vaccine required active phagocytosis, acidification of endosomal compartments, selective use of lysosomal enzymes, such as calpains and cysteine proteases, and the peptide transporter TAP. Cross-presentation with NY-ESO-1 ISCOMATRIX vaccine, however, occurs largely independently of the traditional proteosome and primarily via tripeptidyl peptidase II [19]. Importantly, DCs pulsed with NY-ESO-1 ISCOMATRIX vaccine exhibited prolonged antigen presentation, which efficiently stimulated NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells for up to 3 days, which was the last timepoint examined in this study. We have been able to show *in vivo* that this prolonged presentation also occurs in the draining lymph node of mice (data not shown). Prolonged presentation such as this increases the potential for productive DC and lymphocyte interactions.

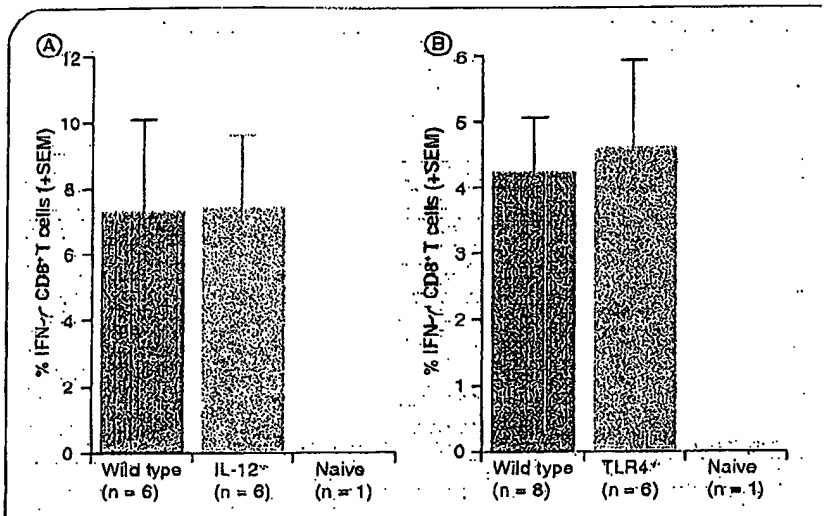

Figure 5. ISCOMATRIX™ adjuvant does not require IL-12 or TLR4 for optimal T cell immune induction. Groups of mice were immunized on days 0 and 7 with 6 µg of ISCOMATRIX adjuvant and 30 µg of ovalbumin (OVA) protein. On day 14 the CD8+ T cell response to the OVA peptide SIINFEKL was assessed by intracellular cytokine staining.

Furthermore, the ability to access different antigen-processing pathways may increase the breadth of peptides and hence the diversity of CD8$^+$ T-cell response that can be generated in response to ISCOMATRIX vaccines.

Immune responses of ISCOMATRIX vaccines in animal models

ISCOMATRIX vaccines have been shown to generate consistently strong humoral and cellular immune responses in an extensive range of animal species including nonhuman primates. The immune responses generated in response to vaccination with ISCOMATRIX vaccines have been reviewed recently [3].

Humoral immune responses

Parenteral delivery of ISCOMATRIX vaccines to mice induces a balanced Th1/Th2 cytokine response (i.e., IL-2, IL-4 and IFN-γ) and generates antibodies of all IgG isotypes, including IgG_1 and IgG_{2a}. This is a major advantage over aluminum-based vaccines because it mobilizes a broader range of antibody-mediated effector mechanisms, such as complement activation, viral neutralization, antibody-dependent cell-mediated cellular cytotoxicity, opsonization and phagocytosis [3]. This makes the ISCOMATRIX adjuvant suitable for use in prophylactic vaccines where induction of strong, long-lived antibody responses is generally the goal. Studies in several small-animal models and nonhuman primates have demonstrated a major antigen dose-reduction benefit of the ISCOMATRIX adjuvant for parenterally delivered vaccines. This includes a ten- to 100-fold lower antigen dose requirement in guinea pigs for the generation of neutralizing antibody responses against HIV antigen, gp120, when combined with the ISCOMATRIX adjuvant compared with the same antigen formulated with aluminum hydroxide [22]. In addition, ISCOMATRIX vaccines can achieve acceptable antibody responses with fewer doses than with aluminum-adjuvanted vaccines and the responses generated have increased longevity.

Cellular immune responses

Chronic viral infections

The induction of high frequency, MHC I-restricted, cytolytic CD8$^+$ T cells is thought to be crucial to the successful clearance of most chronic viral infections, (e.g, HBV, HCV, HIV and human papillomavirus [HPV]) [23]. However, a significant hurdle for most therapeutic vaccine candidates for human use is the poor induction of MHC I-restricted CD8$^+$ T cells. Delivery systems, such as DNA and viral vectors, have offered some hope but have potential safety concerns, and in the case of DNA, generally elicit poor CD4$^+$ and CD8$^+$ CTL responses. In addition, many of these viral vector strategies induce neutralizing antivector antibodies, limiting their repeated use. Prime–boost combinations of DNA and live viral vector delivery are currently being evaluated and although results have been promising in animal models, they are yet to be demonstrated convincingly in humans. Several animal models demonstrate ISCOMATRIX vaccines to be potent inducers of both CD4$^+$ and CD8$^+$ T-cell responses to a wide variety of antigens, such as naturally occurring immunogens, recombinant proteins, peptides [24] and multiple MHC I epitopes arranged in a linear array, referred to as a POLYTOPE™ vaccine [25]. In a rhesus macaque study, a HCV core ISCOMATRIX vaccine induced strong CD4$^+$ and CD8$^+$ T-cell responses against a broad range of epitopes in 100 and 60% of vaccinated animals, respectively [26]. Furthermore, the HCV core ISCOMATRIX vaccine generated long-lived, CD8$^+$ CTL responses, which were still detectable at a high magnitude almost 1 year after the final dose. Conversely, CTL responses induced with recombinant vaccinia virus expressing HCV core protein were diminished by 4 weeks and negligible by 18 weeks postvaccination. Interestingly, ISCOMATRIX vaccines are also capable of inducing CD8$^+$ T cells in the absence of CD4$^+$ T-cell help [12] and CD40 ligation (see later). The mechanisms by which this occurs are not well understood but are the focus of current studies by our group. The capacity to induce a CD8$^+$ CTL response independent of CD4$^+$ T-cell help potentially offers an advantage in the settings where CD8$^+$ T-cell responses are required in a patient population with impaired CD4$^+$ T-cell function (e.g., chronic viral infections, such as HIV, as well as certain solid cancers).

Tumor immunotherapy

As with chronic viral infections, the generation of strong tumor-specific CD8$^+$ CTL responses are critical if vaccine-based cancer immunotherapy is to be successful. In this

regard, ISCOMATRIX vaccines have been shown to protect mice against subsequent challenge with a variety of tumor models including EG7 (EL4 thymoma cells expressing OVA), B16-OVA, Lewis lung-OVA [12] and B16-NY-ESO-1 [17]. Successful protection in such prophylactic mouse models is an essential prerequisite in the evaluation of vaccine candidates. Although several vaccination strategies have overcome this initial hurdle, few have succeeded in the more challenging therapeutic models, where eradication of established tumor burden is a measure of success. In this regard, a recombinant fusion protein consisting of the E6 and E7 proteins from HPV16 formulated with ISCOMATRIX adjuvant has shown some therapeutic effect in mouse tumor models even after a single immunization (data not shown). In more clinically relevant studies, an ISCOMATRIX vaccine containing the NY-ESO-1 protein (a tumor-associated antigen expressed on a variety of human cancers including melanoma, breast, and colon) [27] has been tested in both mice and human DCs [17]. The NY-ESO-1 ISCOMATRIX vaccine was ingested readily by human monocyte-derived DCs and efficiently processed and presented on both MHC II and MHC I molecules to induce NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells, respectively. This NY-ESO-1 ISCOMATRIX vaccine also induced $CD8^+$ T cells in HLA-A2 transgenic mice [17], that were capable of recognizing and lysing human HLA-A2$^+$ NY-ESO-1$^+$ tumor cells. However, it is probable that successful therapeutic vaccines will not only be required to effectively deliver the tumor antigen to APCs *in vivo* but also provide the necessary conditioning for licensing these APCs and other innate immune response effectors in order to generate the overwhelming tumor-specific T-cell responses needed for eradication of established disease.

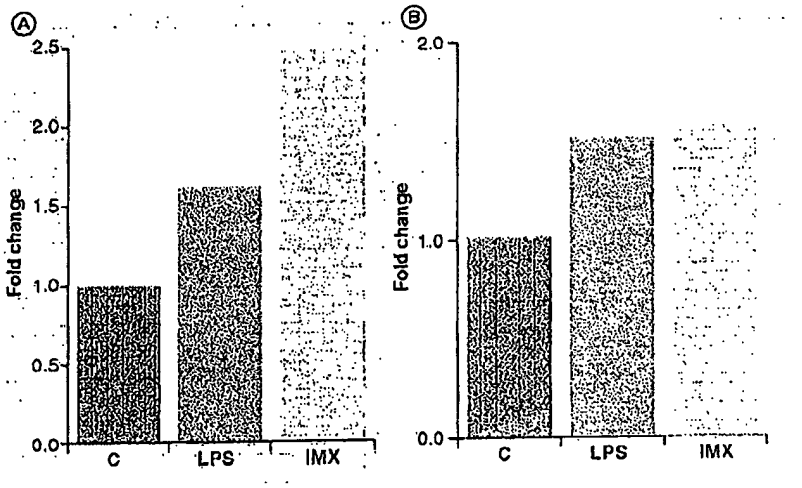

Figure 6. ISCOMATRIX™ adjuvant increases surface expression of markers on dendritic cells *in vivo*. Mice were given 5 µg of LPS or ISCOMATRIX adjuvant subcutaneously and the level of surface expression on dendritic cells in the draining lymph node were determined by flow cytometry. The fold increase in the mean fluorescence intensity of (A) MHC Class II and (B) CD86 markers is shown for CD8 positive conventional dendritic cells.

Clinical trials with ISCOMATRIX vaccines

The optimized ISCOMATRIX adjuvant, in combination with antigen (ISCOMATRIX vaccine), has been studied in seven completed Phase I or II randomized, placebo- or antigen-controlled studies designed to assess safety, tolerability and immunogenicity of the respective vaccines. Intramuscular formulations have been tested in five studies, an intranasal formulation was assessed in one study and pulsed DCs were used in the last. The ISCOMATRIX vaccines tested are part of development programs for either preventative infectious disease indications or therapeutic vaccine programs for infectious diseases or oncology. Within the studies, 198 participants have received at least one dose of an optimized ISCOMATRIX vaccine. Of these, 70% were healthy volunteers, 4% had cancer, 14% were HIV positive and 12% had chronic HCV infection.

At the time of writing, there are seven ongoing studies with an ISCOMATRIX vaccine. To date, no vaccines containing ISCOMATRIX adjuvant have been licensed by any regulatory agency.

The completed clinical trials are summarized in TABLE 1.

Immune responses

The immunogenicity of an ISCOMATRIX vaccine can only be evaluated within the confines of the development program of that vaccine because the immune response is affected by the antigen used, vaccination schedule and study population. The humoral and cellular responses observed with ISCOMATRIX vaccines have previously been described and published [3,28]. In all studies, systemic antibody responses are consistently induced by the ISCOMATRIX vaccine. Analysis of the cellular responses, particularly in the studies assessing the HPV16 E6E7, HCV core protein and NY-ESO-1 antigens, demonstrates that the ISCOMATRIX vaccines induce both antigen-specific $CD4^+$ and $CD8^+$ T-cell responses in the majority of study participants.

While the majority of participants in studies to date have been immunocompetent, there are data suggestive that ISCOMATRIX vaccines are able to induce broad cellular responses in immunocompromised subjects as in the study evaluating the NY-ESO-1 antigen in melanoma patients. Several participants who had received a prototype NY-ESO-1 ISCOMATRIX vaccine in one clinical trial were participants in a subsequent NY-ESO-1 ISCOMATRIX vaccine trial several

Table 1. Summary of completed clinical trials with optimized ISCOMATRIX™ vaccines.

Study number	Clinical program (administration)	Study population	Number*	Number of vaccinations	Dose (µg)		Control
					IMX	*Antigen*	
1	Inactivated influenza vaccine (intramuscular)	Healthy volunteers 60–64 years (n = 110)	55	1	60	15, 45	Commercial influenza vaccine or influenza antigen control
2	HPV16 E6E7 vaccine (intramuscular)	Healthy volunteers 18–45 years (n = 42)	36	3	60, 120	5, 25, 70 240	PBS
3	HPV16 E6E7 vaccine (intramuscular)	HIV positive 18–60 years (n = 35)	28	3	120	25, 70 240	PBS
4	HCV core vaccine (intramuscular)	Healthy volunteers 18–45 years (n = 30)	24	3	120	5, 20, 50	PBS
5	HCV core vaccine (intramuscular)	HCV positive 18–60 years (n = 33)	23	3	120	5, 50	PBS
6	Autologous peripheral blood-derived dendritic cells pulsed with NY-ESO-1 vaccine (intradermal)	Patients with treated cancer and minimal residual disease, over 18 years of age (n = 8)	8	3	120	200	Nil
7	Inactivated influenza vaccine (intranasal)	Healthy volunteers 18–45 years (n = 40)	24	2	100, 500, 1000	30	PBS or commercial influenza vaccine

*Number of participants receiving ISCOMATRIX vaccines and does not include those who received placebo or antigen control vaccines.
HPV: Human papillomavirus; IMX: ISCOMATRIX adjuvant; PBS: Phosphate-buffered saline.

years later. When examining their pre-existing T-cell responses to NY-ESO-1 prior to beginning vaccination in the second trial, several of these participants demonstrated a high frequency of MHC I-specific memory $CD8^+$ T-cell responses to several NY-ESO-1 epitopes 500–800 days after completing the first study. This suggests that ISCOMATRIX vaccines generate prolonged memory T-cell responses, which may be of clear benefit for protection against relapse or reinfection.

There are also promising data from a study evaluating a therapeutic ISCOMATRIX vaccine in 35 HIV-positive participants with a baseline $CD4^+$ of greater than 300 cells/µl who had anal oncogenic HPV infection detected by PCR. Participants were randomized as four active to one placebo into one of four cohorts with 28 participants, each receiving three doses of vaccine (HPV16 E6E7 antigen with 120 µg ISCOMATRIX adjuvant) and seven receiving placebo (PBS). The first three cohorts evaluated three ascending antigen doses and the fourth evaluated the effect of accelerated dosing schedule at the highest antigen dose. The data suggest that vaccination was effective as antigen specific IgG antibodies were detected in 96.4% of evaluable participants (27/28). IFN-γ responses as assessed by QuantiFERON™-CMI assay using whole blood stimulated with 5 µg/ml HPV16 E6E7 protein, indicated antigen-specific T-cell activation with approximately 90% (25/28) of evaluable participants responding to active vaccination on at least one postvaccination timepoint. Further detailed evaluation of the immunogenicity data is underway. The vaccine was well tolerated in this population and there were no dose-limiting toxicities or discontinuations due to vaccine-related adverse events. No vaccine-related serious adverse events occurred.

Thus, in clinical trials, ISCOMATRIX vaccines have been both safe and well tolerated by human patients, as well as being highly immunogenic, generating potent antibody as well as broad-spectrum and long-lived $CD4^+$ and $CD8^+$ T-cell effectors. These are all key characteristics required for successful therapeutic vaccines.

Safety & tolerability

The safety and tolerability of vaccines containing QS21 have been described [8,29,30]. General concerns with these vaccines have included increased local and systemic reactogenicity and the potential for induction of immunopathologic adverse reactions. In the clinical trials performed to date using the optimized ISCOMATRIX adjuvant, comprehensive evaluations of safety and tolerability have been carried out and the respective ISCOMATRIX vaccines have been found to be safe and tolerable.

To date, no vaccine-related serious adverse events or deaths have been reported in any completed or ongoing studies. With the optimized ISCOMATRIX adjuvant, no participants discontinued due to adverse events. Although a direct comparison with the prototype ISCOMATRIX adjuvant was not performed, it is interesting to note in studies in which 774 participants were administered prototype adjuvant, 15 withdrew from their

Table 2. Number of participants with solicited and unsolicited injection-site adverse events by maximum intensity reported at any timepoint in the integrated safety population.

Treatment group[a]	Maximum intensity of adverse experience n (%)				Overall[a] n (%)
	None	*Mild (grade 1)*	*Moderate (grade 2)*	*Severe (grade 3)*	
Inactivated split-virion influenza vaccine single vaccination study[b]					
0 µg IMX, antigen control (n = 55)	30 (55%)	19 (35%)	4 (7%)	2 (4%)	25 (45%)
60 µg IMX + influenza antigen, (n = 55)	9 (16%)	32 (58%)	13 (24%)	1 (2%)	46 (84%)
HPV16 E6E7 and HCV core three-vaccination studies combined[c]					
0 µg IMX, placebo saline control, n = 12	4 (33%)	7 (58%)	1 (8%)	0 (0%)	8 (67%)
60 µg IMX + HPV E6E7 antigen, postvaccination, 1, n = 12	0 (0%)	5 (42%)	3 (25%)	4 (33%)	12 (100%)
120 µg IMX + HPV E6E7 or HCV core antigen, postvaccination 1, n = 48	1 (2%)	16 (33%)	16 (33%)	15 (31%)	47 (98%)
60 µg IMX + HPV E6E7 antigen all doses, n = 12	0 (0%)	2 (17%)	1 (8%)	9 (75%)	12 (100%)
120 µg IMX + HPV E6E7 or HCV core antigen, all doses, n = 48	0 (0%)	4 (8%)	18 (38%)	26 (54%)	48 (100%)

[a]Grade 1, 2 or 3 intensity.
[b]TABLE 1, study 1.
[c]HPV16 E6E7 antigen was administered with either 60 or 120 µg IMX. HCV core antigen was only administered with 120 µg IMX. Data for HPV16 E6E7 antigen and HCV Core antigen administered with 120 µg IMX have been combined for the purposes of this analysis (TABLE 1, studies 2 and 4, respectively).
HPV: Human papillomavirus; IMX: ISCOMATRIX adjuvant.

respective study or required early termination of the vaccination schedule or dose reduction due to an adverse event. This suggests that the optimized ISCOMATRIX adjuvant is better tolerated.

A comparison of safety data from three of the seven completed clinical studies provides insight to the safety profile. These three were designed as Phase I safety studies in healthy volunteers to assess the safety and tolerability of the respective ISCOMATRIX vaccines using optimized ISCOMATRIX adjuvant as an intramuscular formulation. Adverse event data were collected and analyzed in a consistent manner across the three studies. Only one serious adverse event, in a participant randomized to ISCOMATRIX adjuvant, was reported. This event was not considered vaccine related.

TABLES 2 & 3 reflect the number of participants with injection site or systemic adverse events experienced at any timepoint in these three studies. In all three studies solicited injection-site reactogenicity included pain, redness and swelling. Severe events were classified as pain limiting normal activity, redness of greater than 50 mm or swelling of greater than 50 mm. Systemic symptoms included fever, headache, myalgia, chills, sweating, nausea, vomiting and fatigue, which were considered severe if participants were unable to work or do usual activity.

In the influenza study (TABLE 1), which was not powered to detect differences in reactogenicity between adjuvant vaccine and the antigen comparators, a trend for a higher proportion of participants receiving adjuvant to experience injection site and systemic adverse events compared with antigen alone was noted. The ISCOMATRIX vaccine and the antigen controls were well tolerated and of those reporting symptoms, in all treatment groups, the majority reported mild injection site and systemic events. Notably the adjuvant did not increase the propensity for severe injection-site reactions or severe systemic events.

In both the HPV16 E6E7 and HCV core protein studies (TABLE 1) a three-dose vaccination regimen was used. Comparisons were made of reactogenicity post the first vaccination and after all three vaccinations. Consistent with other studies, pain was the most commonly reported injection site event reported.

In these two studies, more participants experienced a severe reaction after one vaccination as compared with the influenza study. The severe events were mostly redness and swelling, rather than pain (data not shown), and may have been due to formulation differences as the HPV16 E6E7 vaccine contained urea, which is known to be a local irritant. There was no apparent effect of adjuvant dose on the proportion of participants reporting an injection-site event following administration of the first vaccination.

Systemic events were mostly mild to moderate in intensity and there was no apparent effect of ISCOMATRIX adjuvant dose on the systemic profile in this data series. The most common systemic events reported were self-limiting myalgia and fatigue.

A higher proportion of subjects experienced a severe injection site or systemic event after exposure to three vaccinations. A review of the data revealed this was due to increased opportunity for an event with three exposures and was not due to an accumulative reactogenicity with each subsequent exposure.

In a *post hoc* analysis of the three studies, adverse events of special interest, suggestive of allergic phenomena, were clustered to assess for any evidence of immunopathologic events. No safety signal was evident and no events suggestive of anaphylaxis or an allergic syndrome were reported.

Exploratory assessment of serum markers of autoimmunity, inflammation and allergy from the two multivaccination studies using HPV16 E6E7 and HCV core protein was undertaken. Of note, an onset of positive anticardiolipin antibodies was noted in 0% of placebo and 6% of adjuvant recipients. The significance of this is unclear as this is a nonspecific marker of autoimmunity and can be confounded by concurrent infection. It is possible that an upper respiratory infection experienced by a number of participants may have been the cause. The more sensitive marker anti-B2 glycoprotein I was also tested for and was negative in all participants. Of interest, raised IgE, generally twofold or less over baseline, was noted in 3.6% of participants compared with 8% receiving placebo. None of these participants reported adverse events suggestive of allergy or had raised eosinophil counts.

Minor fluctuations in all laboratory parameters do occur after exposure to ISCOMATRIX adjuvant with or without antigen compared with placebo. However, there does not appear to be a clinically relevant difference in the incidence of laboratory abnormalities where comparisons with an antigen control arm are available.

Of note in this data series, mild transient decreases in platelet counts between 125 and 140 x 10^9/l which were not considered clinically significant were noted in two participants, both of whom received 120 µg ISCOMATRIX adjuvant (with antigen). Similar changes were not noticed with 60 µg, antigen control or placebo. In earlier studies using a prototype formulation of adjuvant, there were two incidences of significant thrombocytopenia although the participants remained clinically asymptomatic and their platelet counts spontaneously improved.

Mild changes occurring in liver transaminases and total bilirubin have been noted which appear to occur more frequently after exposure to the higher dose of 120 µg ISCOMATRIX adjuvant compared with placebo. A similar incidence of such abnormalities has been noted when influenza antigen has been used as control.

The relevance of these as markers of possible safety concern remains unclear and CSL continues to monitor all laboratory parameters in its clinical programs.

Expert commentary & five-year view

To date, very few vaccine adjuvants have been used in registered human vaccines and, in fact, only aluminium adjuvants are used widely. Aluminium adjuvants have proven effective for the induction of humoral immune responses with vaccine antigens that in and of themselves are relatively immunogenic. The advent of recombinant DNA technology has allowed the development of specific antigens for use in vaccines, which ensure products are better defined and, in some cases, permit their production for the first time. This can lead to improved characterization and safety of products due to lack of potentially virulent or carcinogenic components and reduction of impurities. The

Table 3. Number of participants with solicited and unsolicited systemic reactions by maximum intensity reported at any timepoint in the integrated safety population.

Treatment group	Maximum Intensity				Overall[a] n (%)
	None	*Mild(Grade 1)*	*Moderate (Grade 2)*	*Severe (Grade 3)*	
Inactivated split-virion influenza vaccine single vaccination study[b,†]					
0 µg IMX, antigen control, n = 55	14 (26%)	25 (46%)	10 (18%)	6 (11%)	41 (75%)
60 µg IMX + influenza antigen, n = 55	4 (7%)	34 (62%)	12 (22%)	5 (9%)	51 (93%)
HPV16 E6E7 and HCV core three-vaccination studies combined[b]					
0 µg IMX, placebo saline control, n = 12	0 (0%)	6 (50%)	4 (33%)	2 (17%)	12 (100%)
60 µg IMX + HPV E6E7 antigen, postvaccination 1, n = 12	0 (0%)	6 (50%)	5 (42%)	1 (8%)	12 (100%)
120 µg IMX + HPV E6E7 or HCV core antigen, postvaccination 1, n = 48	4 (8%)	21 (44%)	15 (31%)	8 (17%)	44 (92%)
60 µg IMX + HPV E6E7 antigen all doses, = 12	0 (0%)	3 (25%)	7 (58%)	2 (17%)	12 (100%)
120 µg IMX + HPV E6E7 or HCV core antigen, all doses, n = 48	0 (0%)	4 (8%)	24 (50%)	20 (42%)	48 (100%)

[a]Grade 1, 2 or 3 intensity.
[b]TABLE 1, study 1
[†]HPV16 E6E7 antigen was administered with either 60 or 120 µg IMX. HCV antigen was only administered with 120 µg IMX. Data for HPV16 E6E7 antigen and HCV antigen administered with 120 µg IMX have been combined for the purposes of this analysis. (TABLE 1, studies 2 and 4, respectively).
HPV: Human papillomavirus; IMX: ISCOMATRIX adjuvant.

downside, however, is that these antigens are often not very immunogenic and, therefore, require better adjuvants to be effectively used in vaccines. Therefore, there is clearly a need for novel adjuvants and the ISCOMATRIX adjuvant has the features and properties required to be one of the adjuvants used in both prophylactic and therapeutic human vaccines of the future.

To date, there are no vaccines with ISCOMATRIX adjuvant registered for use in humans, although there are a number of effective veterinary vaccines licensed using saponin-based adjuvants using similar technologies. In the human vaccine field, saponin-based adjuvants are in Phase III studies and ISCOMATRIX vaccines have advanced to Phase II studies. The optimized ISCOMATRIX adjuvant is highly characterized, is able to be produced reproducibly at commercial scale and has displayed an excellent immunogenicity and safety profile in clinical trials. As a result, we would expect that either CSL or one of its commercial partners will progress an ISCOMATRIX vaccine to registration within the next 5 years.

Over the next few years, we plan to understand more fully the mechanism of action of the ISCOMATRIX adjuvant, which may lead to a greater understanding of ways to maximize opportunities for the safe and effective use of this vaccine adjuvant. Another area of keen interest is to combine ISCOMATRIX adjuvant with other adjuvants for situations where one mechanism is not sufficient to induce the required immune response, such as in cancer immunotherapy. Studies have already shown that combining ISCOMATRIX adjuvant with oligonucleotide sequences significantly enhance the induction of cytokines, such as IFN-γ, which in turn contribute to induction of both innate and specific immune responses. Further studies are required to understand the effects of combining adjuvants but it is possible that over the ensuing years, knowledge will be sufficient to be able to rationally design not only antigens but also adjuvants to give the desired immune response. The ISCOMATRIX adjuvant will be an integral component in the development of novel human vaccines and either alone or in combination with other adjuvants will facilitate manipulation of the body's own immune system to prevent and/or treat diseases that to date have been refractory to vaccination.

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Key issues

- The optimized ISCOMATRIX™ adjuvant is suitable for use in licensed human vaccines.
- ISCOMATRIX adjuvant is very stable and can be formulated with antigen in a variety of ways to produce ISCOMATRIX vaccines.
- Immune responses induced by ISCOMATRIX vaccines include broad antibody responses as well as both $CD4^+$ and $CD8^+$ T-cell responses.
- ISCOMATRIX adjuvant stimulates innate immune responses and is very efficient at antigen delivery to both the class I- and class II-processing pathways.
- ISCOMATRIX vaccines are safe and generally well tolerated in humans.

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Affiliations

- *Debbie Drane*
CSL Limited, 45 Poplar Road, Parkville, 3052 Victoria, Australia
Tel.: +61 393 891 902
Fax: +61 393 882 063
debbie.drane@csl.com.au
- *Charmaine Gittleson*
CSL Limited, 45 Poplar Road, Parkville, 3052 Victoria, Australia
Tel.: +61 393 891 209
Fax: +61 393 882 351
charmaine.gittleson@csl.com.au
- *Jeff Boyle*
CSL Limited, 45 Poplar Road, Parkville, 3052 Victoria, Australia
Tel.: +61 393 891 694
Fax: +61 393 891 610
jeff.boyle@csl.com.au
- *Eugene Maraskovsky*
CSL Limited, 45 Poplar Road, Parkville, 3052 Victoria, Australia
Tel.: +61 393 892 736
Fax: +61 393 891 610
eugene.maraskovsky@csl.com.au

Encl. 2

Enclosure 2.

Reactogenicity of OVAiscoms according to the present invention with different saponin fractions in separate iscom particles, compared to conventional ISCOMs.

Ovalbumin. Ovalbumin was palmitified for ISCOM incorporation according to (Lövgen-Bengtsson and Morein (Methods in Molecular Medicine, Vol. 42: Vaccine Adjuvants: Preparation Methods and Research Protocols. Ed O'Hagan, Humana Press, 2000). Briefly The OVA was reacted with NPS (Palmitic acid hydroxy succinimide ester) at a molar ratio of 40:1 (NPS:OVA).
Saponin stock solutions, MEGA-10, Lipid mixture and general procedure was according to Example 2 (WO 2004/004762). Reaction mixtures were prepared as described in Table 1.

Formulation (weight ratio A:C)	Palmitified OVA	PC/C (10 mg/ml)	QH-A (100 mg/ml)	QH-C (100 mg/ml)
OVAiscom-A	4 mg	26,7 mg	106,6 mg	-
OVAiscom-C	4 mg	13,3 mg	-	40 mg
OVAiscom-AC (83:17)	4 mg	24,4 mg	88,5 mg	6,8 mg

Final concentration of the reaction mixtures adjusted with buffer in order to set PC/C concentration at 1 mg/ml

Ovalbumin ISCOMs (OVAiscom) were produced with the Quillaja Saponaria Molina subfragments QH-A and QH-C described in Example 1 (WO 2004/004762). The following preparations were manufactured; OVAiscom-A (100% QH-A), OVAiscom-C (100% QH-C), OVAiscom-AC (83% QHA and 17% QHC). From OVAiscom-A and OVAiscom-C, was also prepared a mixture composed of 83% OVAiscom-A and 17 % OVAiscom-Cof ISCOMs. The ratio and concentration of the QH-A and QH-C components were determined by HPLC and the OVA content was quantified by aminoacid analysis. The iscom structures were verified by negative staining elctron microscopy.

Reactogenicity study setup.

To assess the toxicity of several OVAiscom formulations. The formulations differ with regard to composition of the saponins Fractions QHA and QHC as well as whether the different saponin fractions are incorporated together in the ISCOM particles or in separate ISCOM particles.

12 groups of 10 Balb/c mice18-20 grams (6-8 weeks) were included in the study. The different formulations, were dosed according to Table 2, by one subcutaneous injection dorsal in the neck region in front of the shoulders. The animals were observed and scored (0 – 3) for clinical symptoms (lethargy) and lethality several times a day until they were sacrificed after 96 hours. Suffering animals were euthanised and considered as succumbed from the product administered. The treatments groups were blinded throughout the study.

Clinical study parameters (24, 48,72 and 96 hours)

- behaviour (lethargy etc)
- weight

- local reactions at the injection site
- lethality

The results of the study is shown in Table 2.
Table 2

Composition (%A:%C) *	Dose (ug)	Lethargy score**	Deaths (within 96 hours)	body weight (% loss)
ISCOM-A (100:0)	50	1,5	0/10	4,0
ISCOM-A (100:0)	100	1,5	0/10	9,8
ISCOM-C (0:100)	50	0,5	1/10	12,1
ISCOM-C (0:100)	100	5	1/10	14,5
ISCOM-AC (83:17)	50	2	0/10	10,8
ISCOM-AC (83:17)	100	6	0/10	15,7
ISCOM-A + ISCOM-C (83:17)	50	0	0/10	5,7
ISCOM-A + ISCOM-C (83:17)	100	2,5	0/10	10,4
Non-treated controls	-	0	0/10	2,3

Conclusions
As shown ISCOM formulations containing saponin fractions QH-A and QH-C formulated in the same particles excert toxicity in the same range as ISCOMs containing 100% of the toxic component QH-C alone. Contrary, the same amount of saponins mixed together but formulated in separate particles do not excert side-effects over the formulation containing 100% of the non-toxic component QH-A.

Encl. 3
1

Enclosure 3 Lethargy

Lethargy scores of the vaccinated individuals are indicating the comfort or the discomfort of the administered formulations. Low scores indicate better comfort. The formulations prepared according to the MATRIX-MIX concept were well-accepted and caused low scores after the s.c. administration i.e. significantly lower scores than those of the MATRIX-C formulations. The MATRIX-C formulations and free forms of fraction C caused maximum scores i.e. discomfort and lethality (Fig. 1).

Studies have been carried out that compares cytotoxic effects of the various quillaja formulations in free and MATRIX forms. Figure 2 and Table 1 shows that MATRIX-A and MATRIX-MIX are virtually non-toxic in contrast to MATRIX-C currently used human clinical trials. Table 1 demonstrates that a distinct correlation between lethargy, mortality and cytotoxicity. It should be noted that fraction-C in a MATRIX-MIX formulation can be given in 13 fold higher doses than given in a solitary MATRIX-C formulation. In Fig 3 it is illustrated that fraction C could be given 2 or 4 higher concentrations to the indicator cells in the MATRIX-MIX formulation than in the MATRIX-C formulation without go down to the LC 50 survival level.

Thus, MATRIX-MIX formulations cause little discomfort reactions, while fraction-C in MATRIX, currently being in human clinical trials, in the present tests cause cytotoxicity in low dose (LC50 3.1 μg/ml), discomfort and death in doses where MATRIX-MIX is virtually non-toxic but very efficient. To note at the doses referred to the Q-MIX formulation (non-purified quillaja saponins) enhance as potently or even more potently the immune response than the toxic MATRIX-C formulations. That is why MATRIX-MIX formulations have great potential to be well-accepted and efficient adjuvants (immune stimulators) virtually free of discomfort.

Table 1.

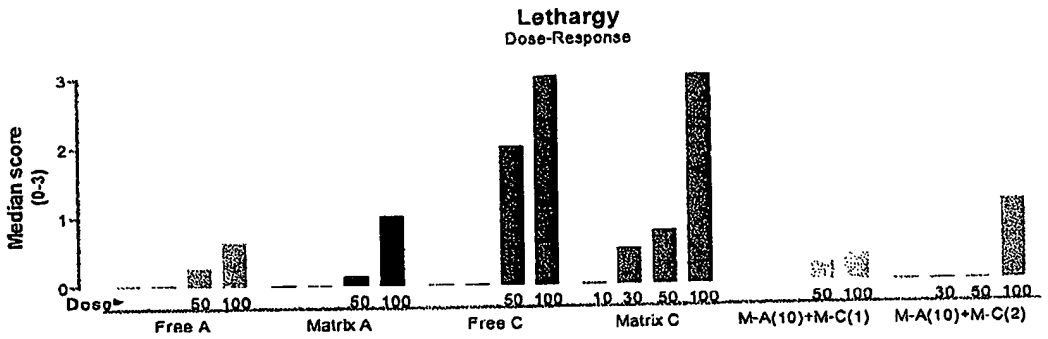
High cytotoxicity correlates to high lethargy and to high mortality

The correlations between *in vitro* and *in vivo* tests

	Free A	Matrix A	Free C	Matrix C	Matrix703	Matrix MIX (Matrix A:Matrix C=1(
Cytotoxicity (LC_{50} µg/ml)	31.836	>1920*	3.291	3.068	18.711	>240*
Lethargy at 100 µg/ mouse (median score)	1	1	3	3	ND	1.125
Mortality100 µg/ mouse	0/8	0/8	3/8	8/8	ND	0/8

*The highest concentration tested

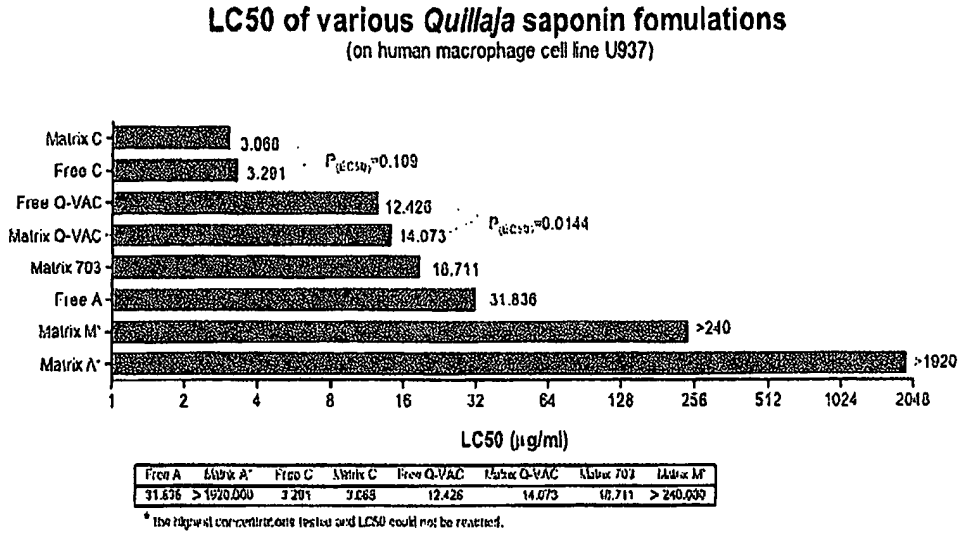
ND: not done. In other animal studies Matrix 703 was more toxic than Matrix C.



Dose (µg)	Free A	Matrix A	Free C	Matrix C	M-A(10)+M-C(1)	M-A(10)+M-C(2)
10				0.000		
30				0.500		0.000
50	0.250	0.125	2.000	0.750	0.250	0.000
100	0.625	1.000	3.000	3.000	0.375	1.125

Fig 1

Dose-response of various quillaja formulations in free or MATRIX forms measured by lethargy in mice after subcutaneous administration as indicated in the figure. The scores are indicated from 0 to 3. Below the graph the scores are in a tabulated form. High scores are indicates discomfort. Low scores indicate low or no discomfort.



Toxicity ranking

1. Matrix C/Free C >3. Free Q-VAC >4. Matrix Q-VAC > 5. Matrix 703 > 6. Free A > 7. Matrix A/AbISCO 100

Fig 2

50% cell death of U932 cells (LC 50) after exposure to various quillaja saponin formulations as indicated in the figure(see tabled information below the figure). While MATRIX C cause LC50 at 3.1 µg the MATRIX MIX and MATRIX A did not cause any cell death at concentrations tested. It should be noted that higher concentrations could not be tested because higher concentrations were not available i.e. 240 µg resp. 1900 µg/ml. Toxicity ranking is also indicated starting with highest cytotoxicity recorded for MATRIX C.

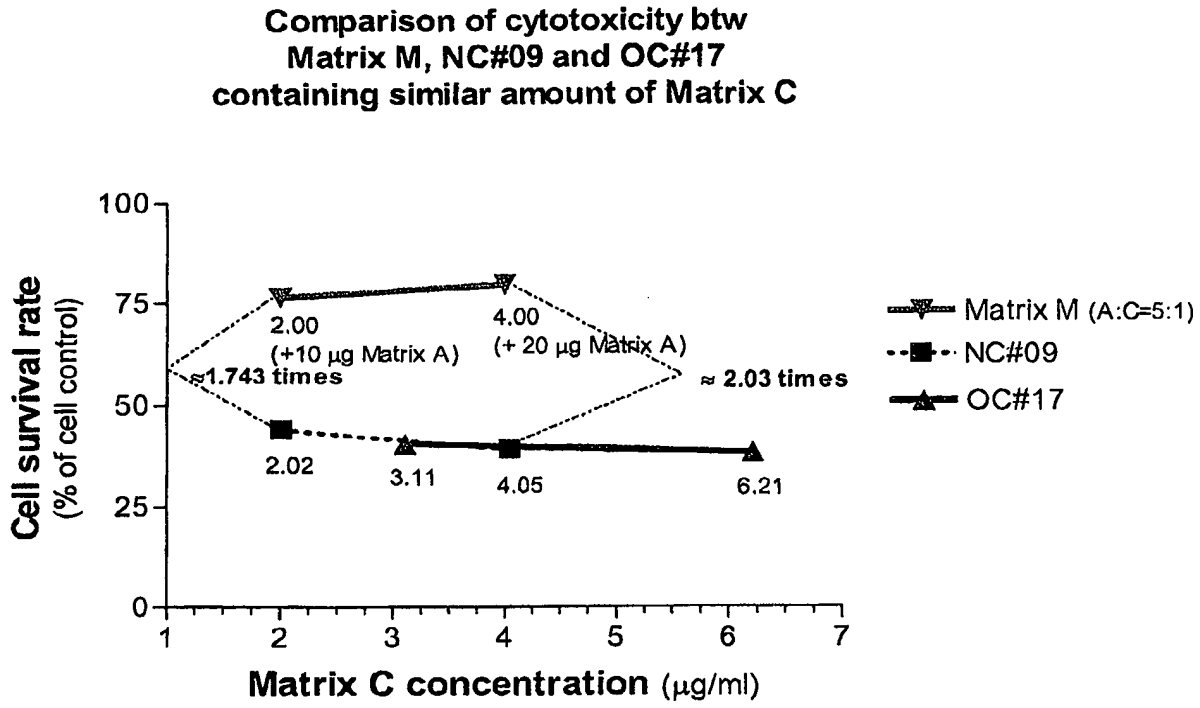


Fig 3

Cell survival measured by percent cell death of U932 cells (LC 50) after exposure to MATRIX MIX and MATRIX C quillaja saponin formulations as indicated in the figure. While MATRIX C cause LC50 at 3.1 µg (see Fig. 2). The MATRIC C formulations are named NC#09 and OC#17. The MATRIX MIX containing10µg MATRIX A and 2µg MATRIX C or 20µg MATRIX A and 4µg MATRIX C did not cause cell death measured by LC50 in doses that exceeded 2 or 4 fold the doses of MATRIX C alone.

End. 4

Acute toxicity in BALB/c mice

Amount*	Matrix formulation (weight ratio)		Lethality#
10 µg	Matrix-A		0/8
50 µg	Matrix-A		0/8
10 µg	Matrix-C		0/8
50 µg	Matrix-C		8/8
50 µg	Matrix-A+C (8/2)	separate	2/8
50 µg	Matrix-A+C (9/1)	separate	0/8
50 µg	Matrix-A+C (9,5/0,5)	separate	0/8
50 µg	Matrix-A/C (7/3)	together	8/8
50 µg	Matrix-A/C (8/2)	together	8/8
50 µg	Matrix-A/C (9/1)	together	6/8
50 µg	Matrix-A/C (9,5/0,5)	together	5/8

* mice were immunized s.c. in the neck region

mice were eutanized or died within 24 h after administration

LowTox-Matrix
Extended acute toxicity in BALB/c mice

Extension of first study

- 15 groups (8/group), BALB/c mice
- Immunisation: subcutaneous (neck) single dose
- Test formulations

• Free Fraction-C and Matrix-C	10, 30, 50, 100 ug
• Free Fraction- A and Matrix-A	50 and 100 ug
• LowTox-MATRIX (9+1)	50 and100 ug
• LowTox-Matrix (10+2)	30, 50, 100 ug
• controls	

- 4 days observation period

LowTox-Matrix
Extended acute toxicity in BALB/c mice

– **Endpoints**

- Primary — **Lethality**
- Secondary — **Lethargy**
  (scores 0 (normal) - 3 (no reaction to external stimuli)

  Gross pathology of liver and spleen
  (scores 0 (normal) - 3 (significant pathology))

LowTox-Matrix
Extended acute toxicity in BALB/c mice

Saponin (weight ratio) ug/dose		lethargy (0-3)*	mortality (%)	liver enlargement/darkness (0-3)		spleen enlargement/darkness (0-3)		gut (0-3)
Fraction-C 100%	50	1.8	38	0,63	1,8	2,1	2,8	(diarrhoea) 1.5
	100	3,0	38	0,64	1,1	2,3	3,0	(diarrhoea) 2.0
Matrix-C 100%	10	0.0	0	0	0	0,0	0	0
	30	0.6	0	0,13	0	0,75	0,65	0
	50	0.6	0	0,12	0,63	2,0	1,63	0
	100	3.0	100	1,0	2,0	2,0	2,4	(diarrhoea) 0,1
Fraction-A 100%	50	0,4	0	0	0,75	0	0,75	0
	100	0,7	0	0,13	0,5	1,13	1,62	0
Matrix-A 100%	50	0,2	0	0	0	0	0,5	0
	100	1,2	0	0,24	0,38	1,5	0,88	0
LowTox (90% A +10% C)	50	0,3	0	0	0	0,12	0	0
	100	0,4	0	0,25	0	0,88	0,25	0
LowTox (83%A+17%C)	30	0,0	0	0	0	0	0	0
	50	0,1	0	0	0	0,12	0	0
	100	1,1	0	0	0	1,62	0,38	0

** mean cumulative scores, accumulated during four days or surviving days*